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Cell endogenous activities of fukutin and FKRP coexist with the ribitol xylosyltransferase, TMEM5

Ryuta Nishihara [a], Kazuhiro Kobayashi [a], Rieko Imae [b], Hiroki Tsumoto [c], Hiroshi Manya [b], Mamoru Mizuno [d], Motoi Kanagawa [a], Tamao Endo [b], Tatsushi Toda [a, e, *]

[a] Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-chou, Chuo-ku, Kobe 650-0017, Japan
[b] Molecular Glycobiology, Research Team for Mechanism of Aging, Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology, 35-2 Sakae-cho, Itabashi-ku, Tokyo 173-0015, Japan
[c] Proteome Research, Research Team for Mechanism of Aging, Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology, 35-2 Sakae-cho, Itabashi-ku, Tokyo 173-0015, Japan
[d] Laboratory of Glyco-organic Chemistry, The Noguchi Institute, Itabashi, Tokyo 173-0003, Japan
[e] Department of Neurology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

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ABSTRACT

Dystroglycanopathies are a group of muscular dystrophies that are caused by abnormal glycosylation of dystroglycan; currently 18 causative genes are known. Functions of the dystroglycanopathy genes *fukutin*, *fukutin-related protein* (*FKRP*), and *transmembrane protein 5* (*TMEM5*) were most recently identified; fukutin and FKRP are ribitol-phosphate transferases and TMEM5 is a ribitol xylosyltransferase. In this study, we show that fukutin, FKRP, and TMEM5 form a complex while maintaining each of their enzyme activities. Immunoprecipitation and immunofluorescence experiments demonstrated protein interactions between these 3 proteins. A protein complex consisting of endogenous fukutin and FKRP, and exogenously expressed TMEM5 exerts activities of each enzyme. Our data showed for the first time that endogenous fukutin and FKRP enzyme activities coexist with TMEM5 enzyme activity, and suggest the possibility that formation of this enzyme complex may contribute to specific and prompt biosynthesis of glycans that are required for dystroglycan function.

1. Introduction

α-Dystroglycan (α-DG) is a cell surface receptor for extracellular matrix proteins, such as laminins and perlecan. α-DG is anchored to the cell surface by the transmembrane protein β-DG, which intracellularly binds to dystrophin. *O*-mannose-type glycosylation is necessary for the ligand binding activity of α-DG (Fig. 1A) and defects in glycosylation are known to cause a group of muscular dystrophies collectively called the dystroglycanopathies [1–3]. Dystroglycanopathies often accompany abnormalities in the central nervous system and eyes [4–6]. To date, 18 dystroglycanopathy-causing genes have been identified [7]. Among them, *fukutin* was originally identified as the causative gene for Fukuyama-type congenital muscular dystrophy [8,9]. *Fukutin-related protein* (*FKRP*), which shares homology with *fukutin*, was identified as the causative gene for congenital muscular dystrophy type 1C and limb girdle muscular dystrophy 2I [10,11]. Defects in *TMEM5* have been reported to cause Walker-Warburg syndrome [12,13].

O-mannosyl glycans that are essential for the ligand-binding activity of α-DG are the CoreM1 and CoreM3 glycans (Fig. 1A) [14]. Repeated units of [-3GlcAβ1-3Xylα1-] serve as the ligand binding moiety, which is modified by the glycosyltransferase LARGE [15]. A tandem ribitol-phosphate (Rbo-P) structure connects CoreM3 and [-3GlcAβ1-3Xylα1-] repeats. Recently, we demonstrated that *fukutin* and *FKRP* encode Rbo-P transferases that are responsible for the synthesis of the tandem Rbo-P structure and that *TMEM5* encodes ribitol xylosyltransferase (RXYL1) to form a Xyl-Rbo-P linkage [16,17]. Some enzymes involved in *O*-mannosyl glycosylation of α-DG are known to form a protein complex. Formation of the POMT1/POMT2 complex is essential to produce protein *O*-mannose transferase activity [18]. POMGnT1 is known to interact with fukutin, presumably to recruit fukutin to its target CoreM3 [19,20]. B4GAT1 is also known to interact with LARGE [21]. Although all enzyme activities required for functional maturation

* Corresponding author. Department of Neurology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
E-mail address: toda@m.u-tokyo.ac.jp (T. Toda).

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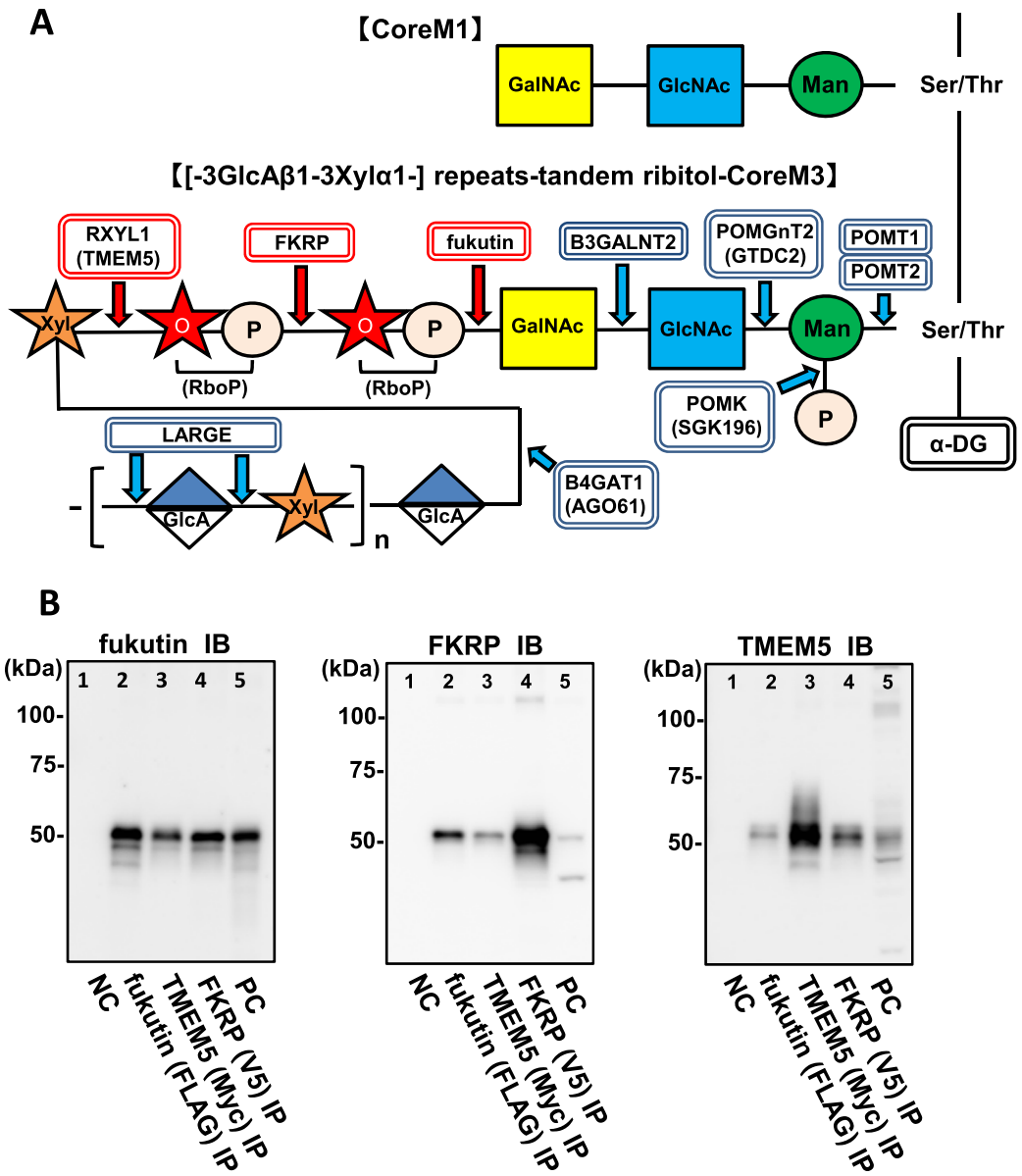

Fig. 1. Interaction between fukutin, FKRP, and TMEM5. A, Schematic representation of the sugar chain structure of α-DG (CoreM1 and [-3GlcAβ1-3Xylα1-] repeats-tandem ribitol-CoreM3), and enzymes responsible for glycosylation. B, Coimmunoprecipitation of fukutin, FKRP, and TMEM5. Fukutin-FLAG, FKRP-V5, and TMEM5-Myc were co-expressed in HEK293T cells. Lysates were subjected to immunoprecipitation with antibodies to FLAG, V5, or Myc. The immunoprecipitated samples were analyzed by Western blotting using antibodies raised against fukutin, FKRP, or TMEM5. Lane 1, total lysate from HEK293T cells without transfection as a negative control (NC); lanes 2–4, immunoprecipitated samples using antibodies to FLAG (lane 2), Myc (lane 3), and V5 (lane 4), respectively; and lane 5, total cell lysate from HEK293T cells expressing fukutin-FLAG, FKRP-V5, or TMEM5-Myc as positive controls (PC).

of dystroglycan have been determined, it is still unclear as to how such complex steps of glycosylation are regulated to be highly specific to dystroglycan. Because fukutin, FKRP, and TMEM5 are enzymes involved in the synthesis of Xyl-Rbo-P-Rbo-P-GalNAc [16,17], we hypothesized that they may exist in close proximity or even form a complex to provide prompt and efficient series of modifications. Our results suggest that fukutin, FKRP, and TMEM5 form a protein complex retaining each proteins' enzyme activity.

2. Materials and methods

2.1. Cell culture and transfection

HEK293T and HEK293 cells were cultured in Dulbecco's Modified Eagle's medium (high glucose) supplemented with 10% fetal bovine serum and penicillin/streptomycin. Transfection of cells was performed using Effectene transfection reagent (Qiagen).

2.2. Immunoprecipitation

Transfected cells were harvested at 72 h after transfection. Cells were lysed with lysis buffer (1% Triton X-100 in PBS with a protease inhibitor cocktail [Nacalai Tesque]). After centrifugation at 9,600 × g for 10 min, the supernatant was obtained and then subjected to immunoprecipitation. The following agarose-conjugated antibodies were used for immunoprecipitation: anti-FLAG agarose (Sigma), anti-V5 agarose (Sigma), and anti-Myc agarose (MBL). The supernatants were incubated with the agarose-conjugated antibodies at 4 °C overnight. After washing 5 times with PBS, the bound materials were eluted with glycine-HCl (pH